

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

**0 285 792
A1**

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 88102867.4

(51) Int. Cl. 4: **C12Q 1/18 , //C12Q1/14,
C12R1/46,G01N33/04**

(22) Date of filing: 26.02.88

(30) Priority: 07.04.87 FI 871512

(43) Date of publication of application:
12.10.88 Bulletin 88/41

(54) Designated Contracting States:
AT CH DE FR GB IT LI NL SE

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(54) **A test set and a process for the determination of antibiotics in milk and a novel streptococcus thermophilus strain to be used therein.**

(57) The invention relates to a test set suitable for the determination of antibiotics in milk and comprising a Streptococcus thermophilus T101 concentrate and a water-based protective agent in a dilution ratio of 4 to 5×10^{-2} . The invention is also concerned with a novel Streptococcus thermophilus T101 strain to be used in the test set and with a process of determining antibiotics in milk by means of said test set.

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A test set and a process for the determination of antibiotics in milk and a novel Streptococcus thermophilus strain to be used therein

The invention relates to a test set suitable for the determination of antibiotics in milk. The invention is also concerned with a novel Streptococcus thermophilus strain to be used in the test set and a process for the determination of antibiotics in milk by means of said test set.

In many situations it is of vital importance to be able to detect the presence of small amounts of antibiotics. This is the case in food industries, for instance, where the increased use of antibiotics and chemotherapeutic substances in the treatment of animals has created a need for a simple, reliable and sensitive process of determination. Since antibiotics are used also in the treatment of dairy cows and since antibiotic residues in milk may both cause health hazards and be disadvantageous for food technological reasons, it is especially important to develop processes suitable for an accurate and rapid screening of milk.

Antibiotic residues in milk are generally detected by microbiological processes which utilize the fact that bacteria are able to produce acid, reduce colours and produce growth on an agar medium. These processes are based on the bactericidal, inhibitory and morphological effect of antibiotics on certain microorganisms.

The Thermocult disk technique is an agar diffusion technique which is widely used in Finland and accepted as an official antibiotic determination procedure. In this technique the test organism is B. stearothermophilus var. calidolactis. It has been developed on the basis of an IDF standard process (IDF 1970. Detection of Penicillin in Milk by a Disk Assay Technique. International Standard FIL-IDF 57. Brussels).

A process of corresponding sensitivity is disclosed by van OS et al., Diffusion Test for the Determination of Antibiotic Residues in Milk. *Neth. Milk and Dairy J.* 29 (1975) 16. The Delvotest process, too, uses B. stearothermophilus var. calidolactis as the test organism. A sample (0.1 ml) is pipetted on agar contained in an ampoule, and a tablet containing nutrients and a pH indicator is added to the ampoule. The process is based on the acid producing capability of the test organism. The ampoules are incubated at 64°C for 2.5 hours. The evaluation is based on the colour change of the agar layer.

Standard techniques further include the Inter-test (BCP-Test). The test microbe used in the process is Str. thermophilus. A test tablet containing a lyophilized culture of the test microbe, nutrients, and a pH indicator (bromocresol purple) is added to a milk sample. The incubation time is 4 hours at

45°C. If the sample does not contain any antibiotic, the colour of the solution turns from blue to green and further to yellow. The amount of the antibiotic can be determined to some extent on the basis of the colour by comparing to a colour map (THOROGOOD et al., An Evaluation on the Charm Test - A Rapid Method for the Detection of Penicillin in milk. *J. Dairy Research* 50 (1983) 185).

A drawback of these processes is their insufficient sensitivity in view of the needs of milk technology.

The determination of antibiotic residues in milk by means of chemical or physico-chemical processes is considerably less usual than the use of micro biological processes. Colorimetric and chromatographic processes require skilled labour and often a complicated and expensive analyzing equipment. The processes are seldom suitable for routine analyses.

The Charm test (CHARM, S.E., A 15-minute Assay for Penicillin and other Antibiotics. *Cultured Dairy Products J.* 14 (1979) 24) is based on the detection of radioactivity. A lyophilized culture of B. stearothermophilus culture and lyophilized ¹⁴C-labelled penicillin are added to a sample. The amount of ¹⁴C contained in the bacterium cells is detected by a Geiger counter; the lower the penicillin concentration of the sample, the higher is the reading of the Geiger counter. The detection time is only 15 minutes and the sensitivity of the process is 0.005 I.U. of penicillin per ml. This process, either, is not suitable for routine use; it is expensive and complicated and requires skilled persons and expensive equipment to be carried out.

Thus there is still a practical need for a sensitive process which is as broad-spectrum as possible. The process should also be simple and it should be possible to carry out the process by an equipment ready for use, whereby the test does not require skilled persons, but can be carried out e.g. on a farm.

These advantages are obtained by means of a test set according to the invention, which is characterized in that it comprises a Streptococcus thermophilus T101 concentrate and a water-based protective agent in a dilution ratio of 4 to 5 × 10⁻². The determination is carried out according to the invention by adding a sample to a test set comprising a Streptococcus thermophilus T101 concentrate and a water-based protective agent in a dilution ratio of 4 to 5 × 10⁻², and possibly an indicator, and if the test set does not comprise an indicator, an indicator is added, too; incubating the test set and the sample at 38 to 42°C for about 4 hours;

TABLE
Experimentally determined antibiotic sensitivities (µg/ml) of the tested determination processes

ANTIBIOTIC	PROCESS ACCORDING TO THE INVENTION		THERMOCULT		DELVOIEST P		INTERTEST b)	CHARM TEST II
	A	B	own deter- mination	(S&S) a)	own deter- mination	(S&S) a)		
PENICILLIN	0.001- 0.002 I.U.	0.001- 0.002 I.U.	0.006-0.0075	0.005- 0.0075	0.0025	0.0025	0.005	0.003
STREPTOMYCIN	1.25	0.25-0.4	5.0	2.5-5.0	5.0	2.5-5.0	5.0	0.1
TETRACYCLINE	0.05-0.1	0.05	0.2 c)	0.1	0.2	0.1	0.5	0.2
OXYTETRACYCLINE	0.1	0.05	0.2 c)	0.1	0.2	0.1	0.2	
AMPICILLIN	0.01	0.003	0.01 c)	0.005	0.01 c)	0.005	0.005	
ERYTHROMYCIN	0.01-0.05	0.01	0.1 c)	0.5-0.75	0.1 c)	0.75-1.0	0.1	0.01
CHLORAMPHENICOL	0.1-0.5	0.1	1.0 c)	7.5	1.0 c)	7.5-10.0	1.0	0.05
NEOMYCIN	0.5	0.1-0.2	0.5	1.0	1.0	0.5	20.0	
STREPTOMAX (penicillin +streptom.)	0.004 I.U. PEN +0.001 µg/ml STREPTOM.		0.01 I.U. PEN +0.008 µg/ml STREPTOM.					
MASTALONE (oxytetra- cycline+the others)	0.1	0.05	0.2 c)					

A indicator added in connection with the test (duration of the test 4 hours)

B indicator added before freeze drying (duration of the test 5 hours)

a) Sandström & Sivelä, 1984, Karjantute 4

b) concentrations given by the manufacturers

c) could not be determined

and evaluating the colour.

The invention is based on the novel Streptococcus thermophilus T101 strain, which has been isolated at the Lammi creamery of Valio. The strain has been deposited at the Deutsche Sammlung von Mikroorganismen under the deposit number DSM 4022 on March 3, 1987, and it possesses the following properties:

- gram positive,
- forms long coccus chains
- growing temperature:
growth at 50°C
no growth at 10°C
- salt resistance
growth at a NaCl concentration of 2%
no growth at a NaCl concentration of 6.5%,
- titrated acidity 25 to 29 °SH after 7-hour incubation at 42°C (sterilized 10% milk powder milk)
- lactic acid %: 0.8% (incubated 2 days at 42°C, from milk powder milk)
- fermentates lactose, saccharose and glucose.

Judging from the values given in the prior art, the novel microorganism strain is clearly more sensitive than known Streptococcus thermophilus strains, especially to penicillin and oxytetracycline.

The test set is prepared in the following way: The microorganism strain is grown in a fermentor at a pH of 6.2 to 6.5 and at 38 to 42°C in a culture medium based on whey permeate. The growth is observed and the growth is arrested at the end of the logarithmic growth phase, whereafter the culture broth is concentrated by filtrating to a 20-fold concentration. The concentrate is measured in a dilution ratio of about 4 to 5 × 10⁻², preferably about 5 × 10⁻², into a protective agent. The protective agent may consist of water-based protective agents used in the preparation of lyophilized microbe preparations. Preferably the protective agent is an aqueous solution comprising 1.1% of sodium glutamate, 1.1% of ascorbic acid, and optionally 7% of lactose, and the pH of which is 6.5. The indicator can be added to the protective agent, or it can be added to the test set in connection with the determination. The indicator is e.g. an acid-base indicator, such as bromocresol purple. The concentrate is measured into a vessel which may be a conventional ampoule, a sealable test tube, a sample bottle, or the like. The vessel is cooled in a carbonic acid-sulphite alcohol bath, whereafter it is lyophilized and stored under vacuum. The finished test set contains about 1 to 2 × 10⁶ bacteria per ml.

The antibiotic determination is carried out by adding a liquid sample and possibly an indicator to the test set. The test set and the sample are incubated and the colour changes are observed. If the sample contains antibiotics, the microorgan-

isms in the test set are not able to grow and the colour does not change. On the other hand, if the sample does not contain antibiotics, the microorganisms grow and induce a colour change while growing.

The sensitivity of the process according to the invention was compared with the corresponding commercial THERMOCULT (Orion Diagnostica) and DELVOTEST P (Gist-Brocades) techniques and the CHARM II technique. The sample consisted of milk preheated at 95°C for 5 minutes and the determinations were carried out according to the instructions given by the manufacturers. The results are presented in the following table, from which further appears the data given by the manufacturer Intervet concerning the INTERTEST. The results show that the process according to the invention is more sensitive than the other processes and detects clearly the presence of all types of antibiotics/combinations.



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EUROPEAN SEARCH REPORT

Application Number

EP 88 10 2867

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 94, no. 21, 25th May 1981, page 561, column 2, abstract no. 172967c, Columbus, Ohio, US; T.M. COGAN et al.: "A rapid method for the detection of penicillin in milk", & IR. J. FOOD SCI. TECHNOL.. 1980, 4(2), 79-88 ---	1,6	C 12 Q 1/18 // C 12 Q 1/14 C 12 R 1/46 G 01 N 33/04
X	DE-A-2 930 394 (S. KOLEHMAINEN et al.) * claims 1,5; page 12, example 13 *	5	
A	---	1,6	
A,D	JOURNAL OF DAIRY RESEARCH, vol. 50, 1983, pages 185-191, London, GB; S.A. THOROGOOD et al.: "An evaluation of the Charm Test - a rapid method for the detection of penicillin in milk" * page 186, paragraph 2 *	1-6	
A	US-A-3 897 307 (R.S. PORUBCAN et al.) * claims 1-5 *	1,2	
A	D.H. BERGEY "Manual of determinative bacteriology", 1974, Williams & Wilkins Co, Baltimore, Md., US; * pages 491-492, 503-504 *	1,5	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			C 12 Q 1/00 G 01 N 33/00
Place of search		Date of completion of the search	Examiner
BERLIN		04-07-1988	DE KOK A.J.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			
I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document			

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Example 1

Preparation of the test set

Bacteria of the Streptococcus thermophilus T101 strain are inoculated in a culture medium having the following composition:

5% of whey permeate powder
1.5% of casein hydrolysate
0.5% of tryptone
1% of yeast extract

The culture medium has been sterilized at 120°C for 15 to 20 minutes, and its pH is 6.4 after the sterilization.

The test strain is grown in a fermentor at a pH of about 6.2 and at about 42°C, and the growth is monitored by observing the turbidity of the culture broth. At the end of the logarithmic growth phase the growth is arrested and the culture broth is concentrated by filtrating using a Millipore Pellicon filtration unit (0.45 μ m) to a 20-fold concentration, whereby the bacterium concentration of the concentrate is about 2×10^9 bacteria per ml. The concentrate is washed with a small amount of protective agent, and about 5 ml is added to 100 ml of protective agent, where to is possibly also added 1 ml of bromocresol purple colour (a 0.8% solution). The bacterium concentration of the solution so obtained is about 1×10^8 bacteria per ml. 1 ml of the solution is added to a conventional 10 ml ampoule which withstands drying and can be closed by vacuum. The ampoule is cooled rapidly (20 to 60 s) in a -60°C carbonic acid-sulphite alcohol bath, whereafter it is freeze dried and vacuum closed for storage. The ampoule thus prepared contains 1 to 2×10^6 bacteria per ml.

Example 2

Determination of antibiotics in a milk sample

Raw milk is heated at 95°C for 5 minutes. 2 ml of milk and possibly 20 μ l of a colour indicator are added to a test set prepared according to Example 1. The test set is incubated for 4 hours at about 42°C and the colour is evaluated. Milk prepared from sour milk powder and heated at 95°C for 5 minutes is used as a control. If the milk contains antibiotics, the colour turns blue. Yellow indicates a negative result.

Claims

1. A test set suitable for the determination of antibiotics in milk, characterized in that it comprises a Streptococcus thermophilus T101 concentrate and a water-based protective agent in a dilution ratio of 4 to 5×10^{-2} .

2. A test set according to claim 1, characterized in that the protective agent comprises an aqueous solution comprising 1.1% of sodium glutamate, 1.1% of ascorbic acid, and optionally 7% of lactose, and the pH of which is 6.5.

3. A test set according to claim 1, characterized in that it further comprises an indicator.

4. A test set according to claim 3, characterized in that the indicator is bromocresol purple.

5. Streptococcus thermophilus T101, DSM 4022.

6. A process of determining antibiotics in milk, characterized by the steps of adding a sample to a test set comprising a Streptococcus thermophilus T101 concentrate and a water-based protective agent in a dilution ratio of 4 to 5×10^{-2} and possibly an indicator, and if the test set does not contain an indicator, an indicator is also added; incubating the test set and the sample at 38 to 42°C for about 4 hours; and evaluating the colour.